# TECHNICAL MANUSCRIPT 80

# ALTERATIONS IN THE BIOLOGICAL ACTIVITY OF PROTECTIVE ANTIGEN OF BACILLUS ANTHRACIS TOXIN

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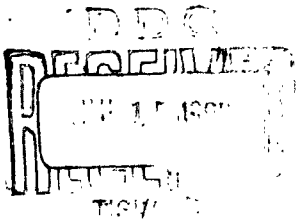

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# ALTERATIONS IN THE BIOLOGICAL ACTIVITY OF PROTECTIVE ANTIGEN OF *BACILLUS ANTHRACIS* TOXIN

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## ABSTRACT

The toxin of Bacillus anthracis contains at least three known components — protective antigen (PA), edema factor (EF), and lethal factor (LF) — that can be assayed by the agar diffusion technique. None of the components is toxic when tested alone, but combination of PA with either EF or LF produces, respectively, the edema reaction (determined in guinea pigs) or lethality (determined as the time to death of Fischer 344 rats).

Several preparations of partially purified PA failed to kill rats when combined with LF, although they were serologically indistinguishable from samples of active PA. Recently this loss in lethal activity has been observed in vivo along with a transient conversion of PA to an inhibitor of toxin lethality. The exact nature of the change or changes is unknown but probably involves subtle physico-chemical changes rather than extensive splitting of the molecule.

The lethality of toxic mixtures was inhibited in vitro by low levels of "altered" PA and by high levels of active PA. These facts are discussed in relationship to the clearance from the bloodstream of injected whole toxin or its components, and a possible role of PA in anthrax therapy is suggested.

## I. INTRODUCTION

The toxin of Bacillus anthracis contains at least three known components — protective antigen (PA), edema factor (EF), and lethal factor (LF) — that can be separated by glass filtration alone[1] or in combination with column chromatography on DEAE cellulose.[2] None of the components is toxic when tested alone, but combination of PA with either EF or LF produces the edema reaction or lethality, respectively.

Strange and Thorne[3] purified the protective antigen and described its chemical and physical properties, which indicate a simple protein molecule. Several preparations of partially purified PA made in this laboratory according to their directions failed to kill rats when combined with LF; however, these preparations were serologically indistinguishable from samples of active PA. Recently this loss in lethal activity has been observed in vivo, along with a transient conversion of PA to an inhibitor of toxin lethality.

## II. MATERIALS AND METHODS

The Sterne strain of B. anthracis was grown under the conditions described previously for toxin production[4] except that the $NaHCO_3$ concentration was increased to 1.2 per cent and was added after four hours' incubation, which frequently increased antigen yields. The toxin was separated into its components by passing culture supernatant fluid through large fritted-glass filters.[1,4]

The components were assayed by the agar diffusion technique of Thorne and Belton,[5] using an antiserum from a horse injected with spores of the Sterne strain of B. anthracis. Toxic activity was determined by the edema reaction in guinea pigs[4] or by the rat lethality test.*[1] Beall et al[1] reported that the time to death in Fischer 344 rats varied in a uniform manner with the amount of lethal material injected. In our experience, this relationship exists only over the range of lethal material killing in approximately 80 to 240 minutes.

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* In conducting the research reported herein, the investigator(s) adhered to "Principles of Laboratory Animal Care" as established by the National Society for Medical Research.

Preparations of PA, partially purified by the method of Strange and Thorne,[3] formed one strong precipitation line and one weaker line in the agar diffusion assay. The stronger line merged with the line produced by preparations of standard PA obtained from Thorne and had a titer of 5000 to 10,000.

Preparations of EF gave two lines in the agar diffusion assay, the weaker one of which merged with the LF line. The stronger line had a titer of 128, and both lines crossed the line formed by PA. Preparations of LF formed only one line in agar diffusion plates and had a titer of 32 to 64. No edema activity was demonstrable on addition of PA to these preparations of LF. In the following presentation, "whole toxin" and a "toxic mixture of PA + LF" are used interchangeably.

## III. RESULTS AND DISCUSSION

### A. CORRELATION BETWEEN CLEARANCE OF TOXIN COMPONENTS FROM THE BLOODSTREAM OF RATS AND LOSS IN LETHALITY

When injected singly into rats, PA or LF is nonlethal, but when injected in combination at appropriate concentrations, rats are killed and the time-to-death response of Fischer 344 rats is proportional to concentration of material injected. Varying the order of timed injections of PA and LF resulted in significant changes in the time-to-death response.

Injection of PA first, followed by injections of either LF or PA + LF (toxic mixture) at the time intervals indicated in Table I, resulted in both a gradual loss in ability of PA to form a lethal mixture with LF and an even slower appearance (about one hour later) of inhibition toward the toxic mixture. This inhibition is manifested as a considerable extension of time to death following injection of the toxic mixture. After 20 to 24 hours, the inhibition was no longer observed, as shown by a return of the time-to-death response to that of the control. PA was no longer detectable in the bloodstream by the agar diffusion assay at about the time it no longer formed a lethal mixture with subsequently injected LF.

On the other hand, when LF was injected first, followed by injections of PA or PA + LF, over a four-hour period there was no significant loss in ability of LF to combine with PA to kill rats, no inhibition of toxin lethality, and no disappearance of LF from the bloodstream. After 20 to 24 hours, LF was no longer demonstrable in the bloodstream, did not form a lethal mixture with PA, and still did not inhibit toxin lethality. In rats injected with lethal mixtures of PA + LF, the LF precipitin line was demonstrable in the blood up to the time the rats died, whereas the PA precipitin line disappeared after about one hour.

TABLE I. CORRELATION BETWEEN CLEARANCE OF TOXIN COMPONENTS FROM BLOODSTREAM AND LOSS IN LETHALITY

| Injection | | Assay Time (min after initial injection) | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Initial | At Time of Assay | 0-30 | 60 | 90 | 120 | 180 | 240 | 20-24 hr |
| | | | | Agar Diffusion Assay | | | | |
| PA | None | + | + | ± | - | - | - | - |
| | | + | + | + | ± | - | - | - |
| LF | None | + | + | + | + | + | + | - |
| | | + | + | + | + | + | + | - |
| PA + LF | None | + | + | (Died, 72 min) | | | | |
| | | + | + | + | (Died, 95 min) | | | |
| | | | | Lethality Assay[a] | | | | |
| PA | LF | 69 | 107 | | S | S | S | S |
| | | 71 | 101 | | S | S | S | S |
| PA | PA + LF | 65 | 78 | | 87 | 148 | S | 66 |
| | | 68 | | | 87 | 151 | S | 68 |
| LF | PA | 75 | 75 | | | 70 | 77 | S |
| | | 75 | 84 | | | 77 | 81 | S |
| LF | PA + LF | | 59 | | | | 63 | 71 |
| | | | 63 | | | | 73 | 71 |
| None | PA + LF | 58 | | | 70 | | 66 | 75 |
| | | 72 | | | 69 | | 66 | 76 |

a. PA and LF were mixed and injected into Fischer 344 rats at 0-, 2-, 4-, and 24-hour intervals. The time to death of each rat is noted in minutes. Simultaneously, the same PA or LF preparation was injected separately, followed by injection of either the other component or a toxic mixture of PA + LF at the time intervals indicated. S = survived.

## B. IN VITRO ALTERATIONS IN PA ACTIVITY

As previously mentioned, several crude preparations of PA obtained as the 28 to 50 per cent $(NH_4)_2SO_4$ fraction did not form toxic mixtures when recombined with EF or LF. The activities of four crude preparations are presented in Table II. All four preparations formed similar lines on agar diffusion against spore antiserum, but only PA 4 and 1 evoked a toxic response upon combination with EF and LF. They were also noninhibitory to toxin lethality. In contrast, PA 3 failed to form active toxin when combined with either EF or LF and it protected the rat from the known killing effect of a toxic mixture. PA 5 also failed to form a lethal mixture with LF but had some edema activity in combination with EF. It was not tested for ability to inhibit toxin lethality. Attempts to alter PA 4 and 1 *in vitro* so that they would exhibit the activities described for PA 3 have so far failed. Complete loss of lethal activity resulted from incubation with trypsin, with rat blood, or with 0.05 M phosphate, pH 7.0/ Evidently milder treatment is required to convert active PA to the inhibitory state.

## C. ABILITY OF PA TO INHIBIT PREVIOUSLY INJECTED TOXIN

Since PA inhibits lethality of subsequently injected toxic mixture, it was of interest to determine if PA could inhibit previously injected toxin. The results in Table III show that, depending on the concentration of toxin, administration of large doses of PA as long as five minutes after toxin injection significantly delayed or prevented death. The significance of the delay in death observed at 15 to 30 minutes is questionable with only one test animal. However, administration of PA at 30 and 60 minutes after toxin injection had no effect on toxin lethality as judged by the time to death following toxin injection.

## D. EFFECT OF THE RATIO OF PA TO LF ON LETHALITY

PA and LF were mixed in various ratios based on their precipitin titers and injected intravenously into Fischer rats at the levels indicated in Table IV. A ratio of PA to LF of 5:4 killed in the shortest time at all levels of LF tested. Increasing the ratio to 5:1 to 10:1 delayed or prevented death, depending on the level of LF. At a concentration of LF of four units per milliliter, none of the rats died regardless of the concentration of PA. Similarly, when the concentration of PA injected was only five units, the rats survived or death was markedly delayed. When serial twofold dilutions of a mixture of PA + LF were injected, the response was comparable to that observed with the corresponding mixtures. Again, levels of five and four units of PA and LF, respectively, were too low to kill.

TABLE II. BIOLOGICAL PROPERTIES OF SEVERAL PREPARATIONS OF PROTECTIVE ANTIGEN

| PA PREPARATION | ACTIVITY | | | |
|---|---|---|---|---|
| | Agar Diffusion Units/ml | Edema[a] + EF, Units/ml | Lethality[b] + LF | + (PA + LF) |
| 3 | 1280 | < 16 | S | S |
| | | | S | S |
| 5 | 640 | 80 | S | |
| | | | S | |
| 4 | 1280 | 2048 | 62 | 68 |
| | | | 65 | 81 |
| 1 | 2048 | 2048 | 71 | 65 |
| | | | 69 | 68 |
| None | | | | 69 |
| | | | | 68 |

a. To measure edema activity, EF was added to serial dilutions of the PA preparation.
b. Eighty agar diffusion units of the PA preparation indicated were mixed with either LF alone or a toxic mixture of PA + LF. Numbers indicate time to death in minutes after injection of these mixtures. S = survived.

TABLE III. ABILITY OF PA TO INHIBIT PREVIOUSLY INJECTED TOXIN

| PA ADMINISTERED | | TIME TO DEATH AFTER INJECTION,[a] MIN | |
|---|---|---|---|
| Units | Time after Toxin | 16 Units Toxin | 32 Units Toxin |
| | Min | | |
| None | - | 100 | 69 |
| 160 | 60 | | 68 |
| 160 | 30 | 100 | 84 |
| 160 | 15 | | 95 |
| 160 | 5 | survived | 210 |
| 160 | 0[b] | | 174 |

a. Each value is the result of a single injection into one Fischer 344 rat.
b. Added to toxin *in vitro* immediately before injection.

TABLE IV. EFFECT OF RATIO OF PA TO LF ON RAT LETHALITY

| RATIO PA TO LF | TIME TO DEATH,[a] min | | | |
|---|---|---|---|---|
| | Concentration of LF, units/ml | | | |
| | 32 | 16 | 8 | 4 |
| 10/1 | S | S | >360 | S |
| 5/1 | | 134 | >180 | S |
| 5/2 | 81 | 89 | 161 | |
| 5/4 | 73 | 85 | 104 | S |
| 5/8 | | 114 | >240 | |
| 5/16 | | >180 | | |
| 5/32 | S | S | | |
| serial twofold dilutions of a mixture at ratio of 5:4 | 72 | 85 | 122 | S |

a. Each of two rats was injected with one milliliter of a given mixture of PA + LF. The values reported represent the mean of 1 to 5 mixtures of each ratio, using different preparations of LF with PA 4 (see Table II).

These results allow several general conclusions to be drawn regarding the *in vivo* fate of injected toxin or its components. Apparently the toxic mixtures of PA + LF as well as PA alone rapidly disappear from the bloodstream of rats and may be fixed at specific tissue sites. If so, absorbed PA not only cannot react with subsequently injected LF to produce the lethal effect, but also undergoes some change that inhibits the lethality of toxic mixtures. The exact nature of this change or changes is unknown, but probably involves subtle physico-chemical changes rather than extensive splitting of the molecule.

The observation that lethality of toxic mixtures can be inhibited by large doses of PA suggests that both are fixed at the same tissue site and that toxic mixtures are fixed through their PA component. The toxin continuously released during anthrax infection might well be counteracted by repeated injections of PA to occupy all the available tissue sites. Since it is generally recognized from the data of Smith *et al*[6] that toxemia is the primary cause of death in anthrax-infected animals, these observations may be useful in exploring a rational therapy in anthrax.

## IV. SUMMARY

Evidence is presented to show that protective antigen undergoes subtle alterations that affect its biological activity. For one hour following intravenous injection of protective antigen (PA) into the rat, the subsequent inoculation of lethal factor leads to death of the rat as expected. Two hours after injection of protective antigen, the injection of lethal factor does not kill the rat, although challenge with whole toxin produces the classical lethal response. At this time, PA is no longer demonstrable in the bloodstream by immunological methods. In contrast, four hours following an injection of protective antigen, rats not only do not die when injected with lethal factor but also are protected from the lethal effect of whole toxin. Some preparations of protective antigen can detoxify *in vitro* whole toxin of known potency. These facts are discussed in relationship to the clearance from the bloodstream of injected whole toxin or its components, and a possible role for PA in anthrax therapy is suggested.

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